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Review of the longevity of the second polar body in the mouse

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Introduction

The polar bodies are derived from meiotic divisions during oogenesis and are contained together with the oocyte within the zona pellucida. Fertilisation triggers the second meiotic division, at which time the second polar body (PB2) is formed (Hogan *et al.*, 1986; Schatten *et al.*, 1988; Johnson & Everitt, 1995) There is no clear evidence on the fate of the polar bodies in any mammal including the mouse, which is the commonly used research model. However, the polar bodies are generally considered as waste material, and therefore not essential to embryo development. In recent years the polar bodies have gained prominence as they have been used in humans for pre-implantation genetic diagnostic purposes (PGD), of single gene disorders, such as determining whether an embryo may have inherited the cystic fibrosis allele from its mother (Munne *et al.*, 1995; Strom *et al.*, 1998; Rechitsky *et al.*, 2000). PB2 also has a potential use in cloning, for the harvesting of stem cells. Wakayama *et al.* (1997) have shown that PB2 has the same genetic potential as the female pronuclei and can be used for the production of normal offspring in mice. The successful use of PB2 for these purposes is dependent on its age, for its longevity, rate and nature of degeneration has yet to be determined. While there is little doubt that the first polar body (PB1) experiences a necrotic fate, the same cannot be said for PB2, which may experience an apoptotic fate. Furthermore if PB2 experiences an apoptotic fate rather than a necrotic one, it would not only be the earliest evidence of apoptosis in a mammal but also provide an excellent research model for the study of apoptosis.

The presence of PB2 is also thought to influence the polarity of the oocyte and zygote, with the location of PB2 determining their long axis (Figs. 1, 2). In such a situation, the longevity of PB2 may well affect any determining influence it has on zygotic and embryonic polarity. Thus determining the longevity of PB2 is relevant to its medical value, its potential research value in apoptosis, and its possible role in influencing the determination of polarity in the early embryo.

In the course of examining the literature and conducting some preliminary investigations I have become aware of the possibility that an aspect of PB2 longevity commonly regarded as fact may be presumption. I refer to the small cell in pristine condition with a conspicuous nucleus seen at first cleavage in the mouse, which is identified as PB2 (see Fig. 3 and Hogan *et al.*, 1986; Johnson, 1988). If it is not PB2, the alternative interpretation is that it is a new cell (produced by an as yet unknown mechanism) and bears only a superficial resemblance to PB2, which it has been mistaken for. Such an interpretation would have considerable ramifications in the areas of cell, reproductive and developmental biology, particularly for invasive techniques such as gene therapy and cloning, which might impair the production of this cell.

The purpose of this review is therefore to examine whether the small cell in pristine condition at first cleavage and well after, interpreted in the literature as being PB2, and therefore implying its longevity past first cleavage, is based on fact or presumption.

Methodology

Central to establishing as fact the assertion that the pristine-looking small cell with the prominent nucleus seen at first cleavage is PB2, is a capacity to demonstrate a continuity of its nuclear DNA over previous stages of the first cell cycle. Does the literature provide evidence of such? Much of the review is devoted to examining this question.

As there are no specific studies on the longevity of PB2 in any mammal, the evidence from the literature, which is cited here, is often peripheral to the main thrust of the publication in which it appears. For this and perhaps other reasons the material is often pre-

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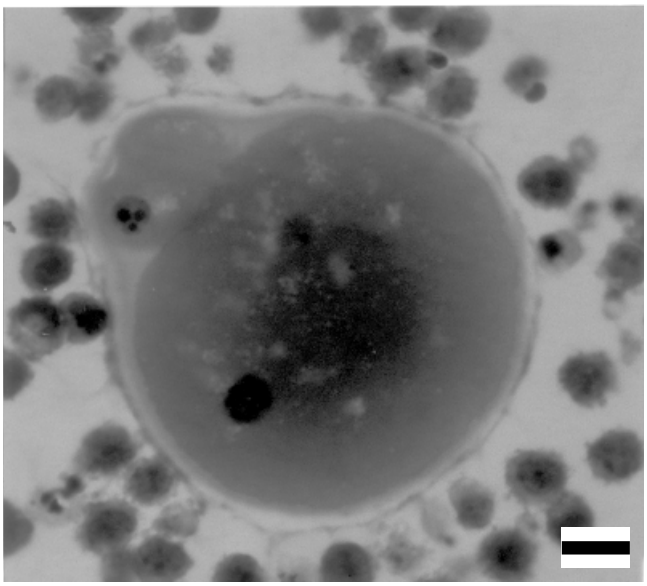

Figure 1 A serial section through an early fertilised oocyte in situ, surrounded by its cumulus mass. The nucleus of PB2 and the male pronucleus are clearly visible. The female pronucleus is closer to PB2 but less distinct due to the plane of section. Scale bar represents 12 µm.

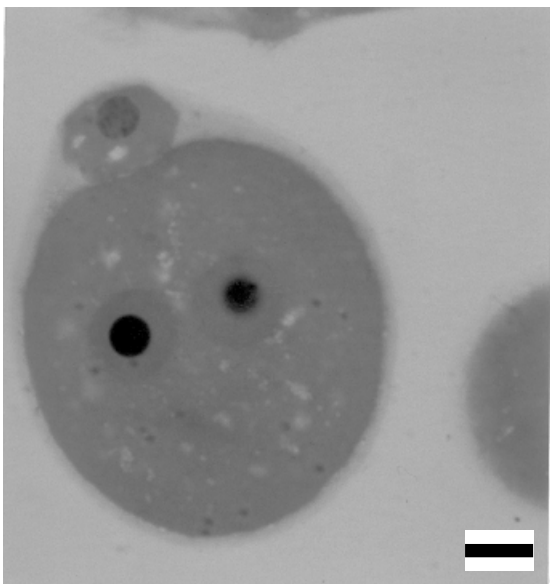

Figure 2 A fortuitous serial section through the nucleus of PB2, male and female pronuclei prior to syngamy of an oocyte *in situ*. The cumulus mass has by now disappeared. The alignment of the pronuclei is approximately at right angles to the long axis of the oocyte, determined by the position of PB2. This orientation is contrary to the conventional understanding, where all three are thought to be in a straight line prior to syngamy (Howlett & Bolton, 1985). Scale bar represents 12 µm.

sented by the authors in such a way that minimal attention is attracted to it – an issue of some concern. Two commercially available videos are also referred to for the quality and time-dependent features of the images.

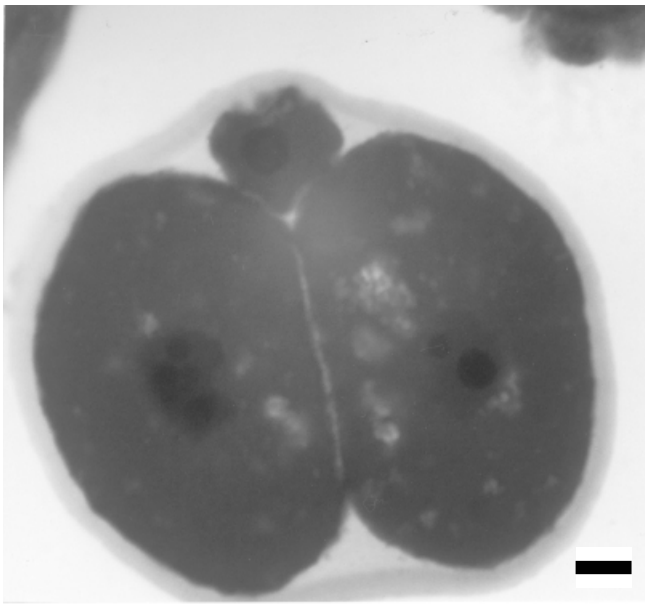

Figure 3 Another fortuitous serial section through a '2-cell' embryo *in situ*. The plane passes through the two blastomere nuclei and the small cell nucleus. The small cell is conventionally presumed to be PB2. Scale bar represents 12 µm.

The figures include both my own (Figs. 1–11) and some from the literature, namely figures from Schatten and colleagues (1988), reproduced with permission. Figures from the latter are referred to with the prefix 'Sch', retaining their original figure reference (e.g., Fig. Sch 3, 1988), and captions. Figs. 1–3 are *in vivo* specimens of fertilised oocytes and a 2-cell embryo from 4 µm serial sections through mouse oviducts, fixed in Karnovsky's fixative, blocked in glycol methacrylate (GMA, 2-hydroxyethyl methacrylate) and stained with 1% toluidine blue. The mice (F1 females of a CBA×C57BL cross, mated to Swiss stud males) were not superovulated and were mated over 30 min periods in the mornings between 08:00 and 09:30 hours. Figs. 4–9 are of *in vitro* specimens, collected after superovulation and overnight matings. The oocytes were cultured in M16 medium (Sigma), in a 4% CO_2 environment and double-stained with Hoechst (33342, Sigma, 2 µl/ml) and propidium iodide (668, Sigma, 10 µl/ml). Hoechst stains both healthy and apoptotic cells, whilst the propidium iodide stains only necrotic cells among living cells, in otherwise untreated tissue. The oocytes and embryos were placed on glass slides with air-dried specimens of mouse hepatocytes and/or chicken erythrocytes as controls for the propidium iodide stain. Figs. 10 and 11 are projections of laser confocal microscope (Biorad MRC 1000/1024UV) images, made of an unfixed air-dried zygote and embryo stained with propidium iodide. Chicken erythrocytes were used as controls.

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Body

The early historical context

Part of the present interpretation that the nucleated small cell seen after first cleavage is PB2 may be due to a change in usage of the term 'polar body'. There appears to have been a progressive shift from its usage as a purely descriptive term meaning something like a small structure peripherally located near polar regions of the egg or embryo, to a term with a particular aetiology. Austin (1961), in his review of the literature, identifies some of the functional roles early investigators attributed to polar bodies. He cites an 1878 account by Blanchard as reporting that these functions included a cushion to protect the vitellus, a form of defecation and rudimentary cells of atavistic significance, and were also widely considered to determine the direction of the cleavage furrow. From this diversity of thinking the predominant defining force to emerge in the conventional usage of the term 'polar bodies' during the twentieth century has not been form or function but aetiology based on Mendelian genetics.

Van Der Stricht in his 1923 report, which contains many illustrations of oocytes, ova, zygotes and embryos of a number of species including mouse, rat, guinea pig, cat and dog, uses the term 'polar body' in a way which begins to entrench such usage. While he does not specifically identify the small cell in the 2-cell embryo as PB2 he nevertheless defines it as '*gp = un des deux globules polaire*', meaning one of the two polar bodies, an aetiological explanation based on Mendelian axioms.

Lewis & Wright's 1935 report may well have been crucial in establishing the basis for the interpretation that the nucleated small cell seen at, and subsequent to, first cleavage was PB2. They reported 'Within 2 h and 40 min several polar bodies were completely separated. The next day, other polar bodies were seen and one egg had divided.' Here the implied comparison is between a newly formed young PB2 and the small cell seen at first cleavage, an event which usually takes place approximately 20 h after mating/insemination, usually in the small hours of the morning, and consequently is not commonly observed. The presumption that the cells are one and the same structure appears to be based on a superficial similarity between the two structures (see Figs. 1–3; Van Der Stricht, 1923; Schatten & Schatten, 1987) and in view of the technology of the time not an unreasonable interpretation. However, the continuation of such a presumption in an era of DNA and cytoskeletal analyses of ova, zygotes and embryos can hardly be justified. The trouble is that when presumptions become engrained in our collective understanding over half a century, there is a tendency for us not only to fail to recognise them as presumptions but they also seem to acquire an almost axiomatic status such that even the most eminent of investigators may either deliberately avoid drawing attention to inconsistencies or develop creative interpretations to accommodate their data within the prevailing dogma.

More recent studies

The first cell cycle

Studies which have either reported direct quantification of PB2 DNA (Howlett & Bolton, 1985) or show qualitative features of its DNA organelles and cytoskeleton (Schatten *et al*., 1986, 1988; Schatten & Schatten, 1987; Schatten *et al*., 1988), fail to show continuity in PB2 DNA over the first cell cycle.

Howlett & Bolton's (1985) report provides measures of DNA content of pronuclei and PB2s. While they provide data on DNA content for the combined pronuclei at sampling times of 8, 10, 12, 14, 16, 18 and 36 h post-insemination (PI), a similar continuous sequence for PB2 is not shown, there being an absence of PB2 DNA data at 12 h and 14 h PI. In the absence of an explanation for this, coupled with their interpretation that the DNA data at 16 h and 18 h PI (see also later comment) is of PB2, the reader may well conclude that the absence of data on PB2 DNA at 12 h and 14 h PI is just one of those investigative quirks and not give it a second thought.

However, Howlett & Bolton's (1985) data could be interpreted differently: that the DNA data at 8 h and 10 h are of PB2 as reported but that the reason for the absence of PB2 DNA data at 12 h and 14 h is that by now the process of PB2 reabsorption has reached the extent that while PB2 may still be represented by a substantial vesicular structure it has lost most if not all of its DNA and what little may remain is too small to be detected by the technique employed. A recent video series presented by Sir Robert Winston shows some excellent time-lapse photography compressed into about a minute or so of an apparently regressing PB2 (The Human Body, 1998). The detection of what appears to be an increasing quantity of DNA between 16 h and 18 h PI could be attributed not to PB2 but to a newly forming small cell which appears to emerge close to the site of final reabsorption of the PB2 vesicle, again visible on commercially available video (Meiosis, 1991; discussed further). These alternative interpretations of Howlett & Bolton's (1985) data are complemented by findings reported in yet other sources, particularly reports emanating from Schatten's laboratory.

Few have investigated the cytoskeletal, organelle and DNA profiles of mouse oocytes, zygotes and embryos as extensively as Schatten and co-workers. Their publications invariably have numerous photo-

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graphic images of stages through the first cell cycle which provide a wealth of material for searches such as this. Often, though, the images are very small, and where enlargements of the central zygotic structures are provided, the corresponding loss of field of view often results in peripheral structures not being visible. Despite their extensive monitoring of DNA in conjunction with cytoskeletal and organelle features of mouse ova over the first cell cycle, none of their reports (cited previously) show a continuity of PB2 DNA over the first cell cycle. The PB2 may be seen in all these reports in the early part of the first cell cycle; occasionally attention is drawn to it by its identification (Schatten *et al*., 1988). No comment is made about discontinuity of its DNA, which though time frames are not provided may, by the locations of pronuclei in relation to syngamy (approximately 16 h PI), be estimated as being just past mid-cycle, thus approximating to the absence of data for PB2 DNA at 12 h and 14 h PI in Howlett & Bolton's (1985) report.

A third source of evidence to support the interpretation that PB2 probably loses most of its DNA after mid-cycle comes from early work in laboratories with an interest in developing a genetic screening technique of ova using PB2. In essence, these reports describe the visualisation of PB2 chromosomes using okadaic acid, confirming the haploid nature of PB2, and are suggestive of its increasing genetic incompetence well before the middle of the first cell cycle (Dyban *et al*., 1992, 1993; Verlinsky *et al*., 1994).

In the light of increasing evidence in the literature, it is difficult to see how the interpretation that an intact PB2 containing DNA, as distinct from a vesicular remnant, continues over the first cell cycle, can be sustained with anything like the confidence it has enjoyed to date. Even more difficult to sustain is the interpretation that PB2 retains a functional nucleus capable of DNA replication (Howlett & Bolton, 1985; Kaufman, 1992), discussed below.

The second cell cycle

How may the presence of the distinctly nucleated small cell in pristine condition in the 2-cell embryo (Figs. 8, 9) be interpreted? Howlett & Bolton's (1985) report, in which they quantify PB2 DNA, shows an apparent increase in the DNA of a structure they identify as PB2. The DNA of this structure appears to increase between 16 h PI and 18 h PI to reach a value of 1.7 (1.0 being the haploid value), though not significantly. No other studies are available against which to compare quantitative measures of DNA content in the small cell at first cleavage. I suspect (based on preliminary findings) that the apparently greater than haploid quantity of DNA in the small cell at first cleavage, reported by Howlett & Bolton (1985), will prove to be the case.

Howlett & Bolton's (1985) report interprets the small cell with apparently increasing quantities of DNA in it as PB2 undergoing DNA replication. Apart from invoking the conventional account that PB2 continues well past the first cell cycle (which it sometimes does but in what appears to be a vesicular form, an issue addressed later), might there be another basis for such an interpretation? If PB1 can divide and it is presumed that this happens after DNA replication (a presumption which may be entirely false for, in the absence of any evidence, it might just as well be argued that such apparent division is little more than a necrotic fragmentation phenomenon), then why should PB2 not at least undergo partial DNA replication and perhaps even sometimes divide? The genetic potential of PB2 has been demonstrated by both Eviskov & Eviskov (1994) and Wakayama *et al.* (1997). Eviskov & Eviskov (1994) first showed that when PB2 chromosomes were transferred to androgenic haploid zygotes they maintained their ability to support normal preimplantation development. However, in their introduction they also state that 'polar bodies never participate in development as they degenerate soon after formation.' It would appear that research on PB2 related both to cloning and PGD would suggest an earlier demise of PB2 than claimed by the traditional conventional account.

How does Schatten's group account for the small cell in the cleaving zygote? With only sparse reference to the phenomenon in general, Schatten & Schatten (1987, p. 344) identified the structure as PB2 on the basis of cytoskeletal attachment to a forming blastomere. Such an interpretation is not surprising given that PB2 has long been known to maintain a substantial connection with the vitellus, the significance of which is poorly understood but the tenacity of which can readily be appreciated by attempting to remove PB2. The assumption here is that such a connection continues into cleavage. Such an interpretation would be quite acceptable provided the structure contained no DNA, being only vesicular (discussed further), for their data also fail to show a continuity or, perhaps one should argue, clearly show a discontinuity in PB2 DNA over the first cell cycle. The alternative interpretation is that the cytoskeletal attachment demonstrated is consistent with a newly forming small cell (discussed further with reference to Figs. Sch 3L, 3O and 4C, 1988).

Kaufman (1992), like Howlett & Bolton (1985), takes the view that PB2 retains a capacity to divide and does so in some strains. His interpretation of the presence of two small cells, one appearing marginally smaller than the other in an 8-cell embryo, is that they have resulted from a division of PB2 and that this may be a strain-related phenomenon. PB1 is not considered as a contender, for it is widely recognised that it undergoes

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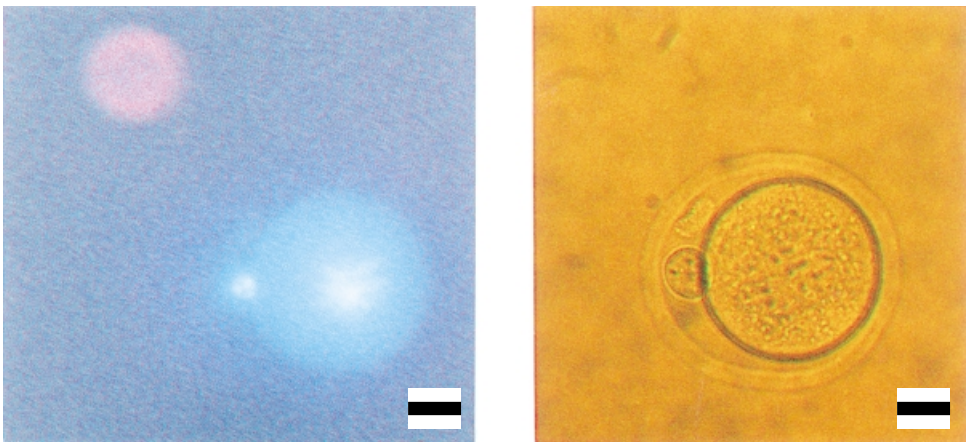
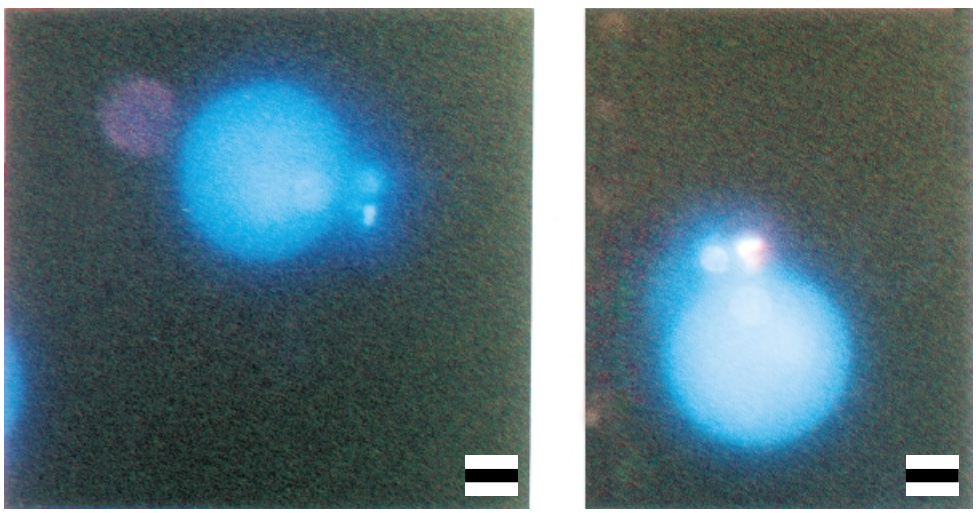
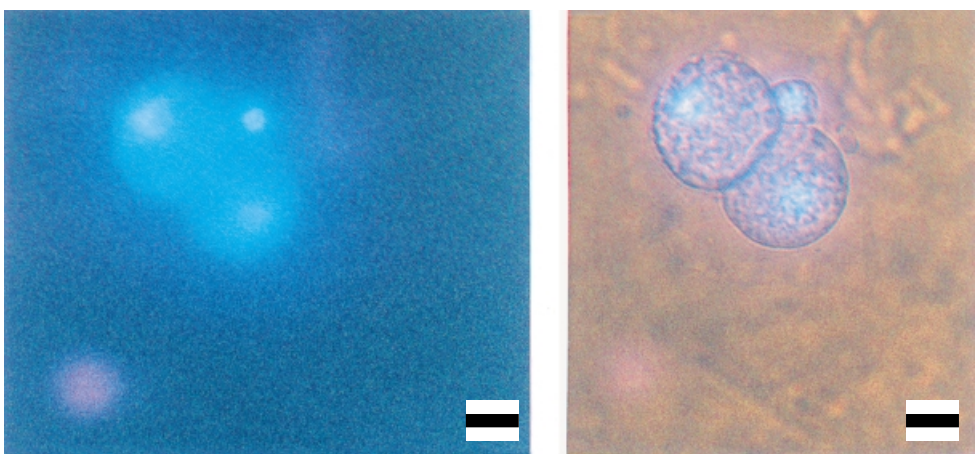


Figures 4, 5 Fluorescent (Fig. 4) and bright-field (Fig. 5) images of the same zygote *in vitro*. PB1 is not visible under fluorescence in Fig. 4, but is visible in bright field in Fig. 5, as a conspicuous structure next to PB2. Under fluorescence PB1 stains neither red with propidium iodide nor blue with Hoechst, indicating it is a residual vesicle which has lost its DNA. The pink-staining sphere on the periphery of the zygote is an air-dried mouse hepatocyte (control). Scale bar represents 18 µm.



Figures 6, 7 A fluorescent image of a recently fertilised oocyte. In Fig. 6, both PB1 and PB2 stain blue with Hoechst. In Fig. 7 a slightly later-stage PB1 stains pink with propidium iodide, indicating a necrotic state. Scale bar represents 18 µm.



Figures 8, 9 Fluorescent (Fig. 8), and a combination of fluorescent and bright-field illumination (Fig. 9), of the same '2-cell' embryo. Just visible in Fig. 9 is a substantial vesicular structure next to the nucleated small cell. In Fig. 8, the same structure does not stain either pink or blue, indicating it contains negligible DNA. Scale bar represents 18 µm.

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necrosis (Figs. 6, 7), disappearing prior to first cleavage. Again as discussed previously it may be argued that if PB1 can divide why not PB2, or given that the female pronucleus has a capacity for DNA replication, why not its sister nucleus in PB2? Such potential capacities may be supported by the previously cited reports of Eviskov & Eviskov (1994) and Wakayama *et al*. (1997). Coupled with these 'potential capacities', are the possibilities of strain variation. However, once again the crucial piece of evidence necessary to support this interpretation is continuity of DNA in PB2 or a daughter cell over the first cell cycle, and this is missing. Perhaps a more plausible alternative interpretation is that while one of these cells was newly formed at first cleavage as appears to be the case in video footage (Meiosis, 1991; Fig. 9), the other was the vesicular remains of PB2, which in these instances was not totally reabsorbed at cytokinesis but continued on past first cleavage. Vesicular remnants are by no means uncommon and can easily be confused for a nucleated cell under bright-field illumination without the use of DNA-specific stains (see Figs. 4, 5, 8 and 9).

Cleavage is probably a very energy-demanding activity, even more so than polar body reabsorption. Daughter blastomeres have the capacity for DNA transcription by about the middle of the second cell cycle, which enables them to influence the composition of their own cytoplasm. Thus, the reabsorption of all the PB2 cytoplasm, in addition to its DNA, may not be of high priority in an evolutionary context of cell energetics. In such a context it is not difficult to envisage how natural selection might lead to strain variation in terms of continuation of the vesicular component of PB2 after first cleavage. Indeed, on this basis one might predict that ova subjected to environmental stress, such as suboptimal culture conditions, would show a higher rate of continuity of PB2 vesicles than non-stressed controls – a testable hypothesis.

What are the difficulties in explaining the source of DNA for a new small cell forming near first cleavage? Convention would dictate that we look to the pronuclei or their post-syngamy complex as the source of origin of this DNA. Applying a creative extension of the norm, one scenario is as follows: The process of DNA replication in each pronucleus proceeds to a 4C state. After syngamy the chromosome complex moves to the periphery, rotating as happens during the formation of PB2 but splitting into two 4C masses. The more peripherally located 4C mass would be incorporated into the new small cell, while the other 4C mass returned to the centre and then divided giving rise to two 2n blastomere nuclei. While this interpretation might appear superficially attractive, partly because it can account for an apparently greater than haploid quantity of DNA in the small cell at first cleavage, it conflicts with other data in the literature. For instance, two reports (Howlett & Bolton, 1985; Winston *et al*., 1993) show DNA content of the two pronuclei just prior to syngamy to be 2n in each. Furthermore, even though cleavage takes place in the small hours of the morning when few are likely to be observing it, it seems unlikely that over half a century the mitotic-like events required by this interpretation would have been missed, for the one thing most scientists excel in is recognising the familiar. However, herein may also lie a crucial weakness: the tendency to expect most phenomena to be explained by the familiar, allowing 'the comfortably familiar to imprison the mind' (Gould, 1989). Without being able to account for the necessary extra DNA production, the traditional conventional approach renders itself impotent to explain the origins of the small cell DNA.

Should we now look for other possible starting points for the DNA and not the pronuclei or their combined mass post-syngamy? This is a major step for any biologist and may be considered a hardly justifiable risk to a professional reputation in terms of the 'throw away' nature of PB2 and the hitherto unrecognised function of the small cell prominent at first cleavage. But let's dare and press on.

Excluding the pronuclei and their post-syngamy mass as potential sources of DNA, there are perhaps only two other options: (a) the production of DNA from a DNA template formed from reabsorbed polar body DNA, and/or (b) the production of DNA by a reverse transcription mechanism from maternally inherited mRNA, of which there are thought to be large quantities in the ovum. The demonstration of either one of these would lead to a small revolution in our thinking on early mammalian cell biology. What evidence, then, is there in the literature to support either of these possibilities? Again some of the best evidence comes from reports emanating from Schatten's group, but first let us explore the potential that such mechanisms could be operative.

There is reason to believe that reabsorbed polar body DNA could act as a template for further DNA synthesis. This may be inferred from reports by Gurdon *et al*. (1974) and a review by Zirkin *et al*. (1989). The latter, in a section on male pronuclear formation and function, argues, citing a variety of reports, that 'numerous investigations have shown that sperm nuclei, somatic cell nuclei , purified DNA , or plasmid DNA, when injected into unfertilised, activated amphibian eggs, serve as templates for DNA synthesis.'

The potential in mammals for reverse transcription of DNA from mRNA is seen in a report by Niu *et al*. (1989), where the transfer of information from mRNA to chromosomes by reverse transcription is reported in a vertebrate, namely in the early development of gold-

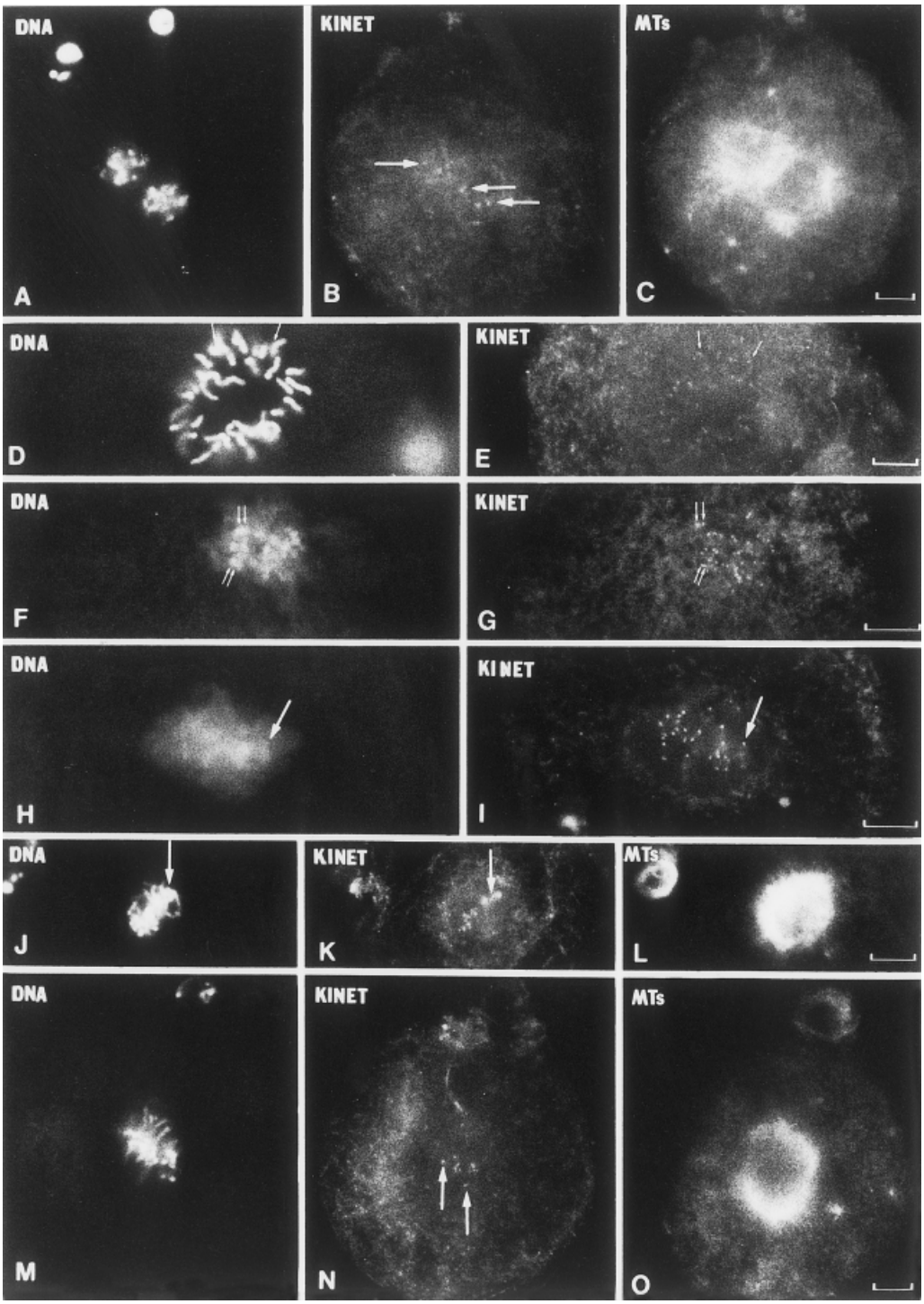

Figure Sch 3, 1988 *'Fig. 3A–O. First mitosis: nuclear breakdown to prometaphase. Pronuclear fusion at fertilization is not found in mammals and instead the nuclear envelopes of the separate but adjacent pronuclei disintegrate at the end of first interphase. At prophase (A), the kinetochores (B) are found as paired structures associated with each condensing chromosome. Later, kinetochores are associated with both the maternal and paternal chromatin mass (D, E) and the cytoplasmic microtubules largely disappear as an irregular microtubule-containing structure emerges around the chromatin mass (C). As the maternal and paternal chromosomes intermix (F, H) and become aligned during prometaphase (G), the kinetochores change their positioning from a wide distribution (G) to a tighter packing (I). At early prometaphase (J) the chromosomes are brought into alignment as the kinetochores (K) are positioned laterally. The mitotic spindle is not yet fully elaborated with an unclear polarity (L). At later prometaphase the chromosomes are better aligned (M) with the kinetochores now at the equator (N) of a spindle (O) with a clearer axis: microtubule cytasters are still apparent (O). A–C and J–L: Triple label for DNA (DNA), kinetochores (KINET) and microtubules (MTs); D–I: double labelled pairs for DNA and kinetochores. Bars represent 10 μm.'* Reprinted with permission from Schatten *et al*. (1988).

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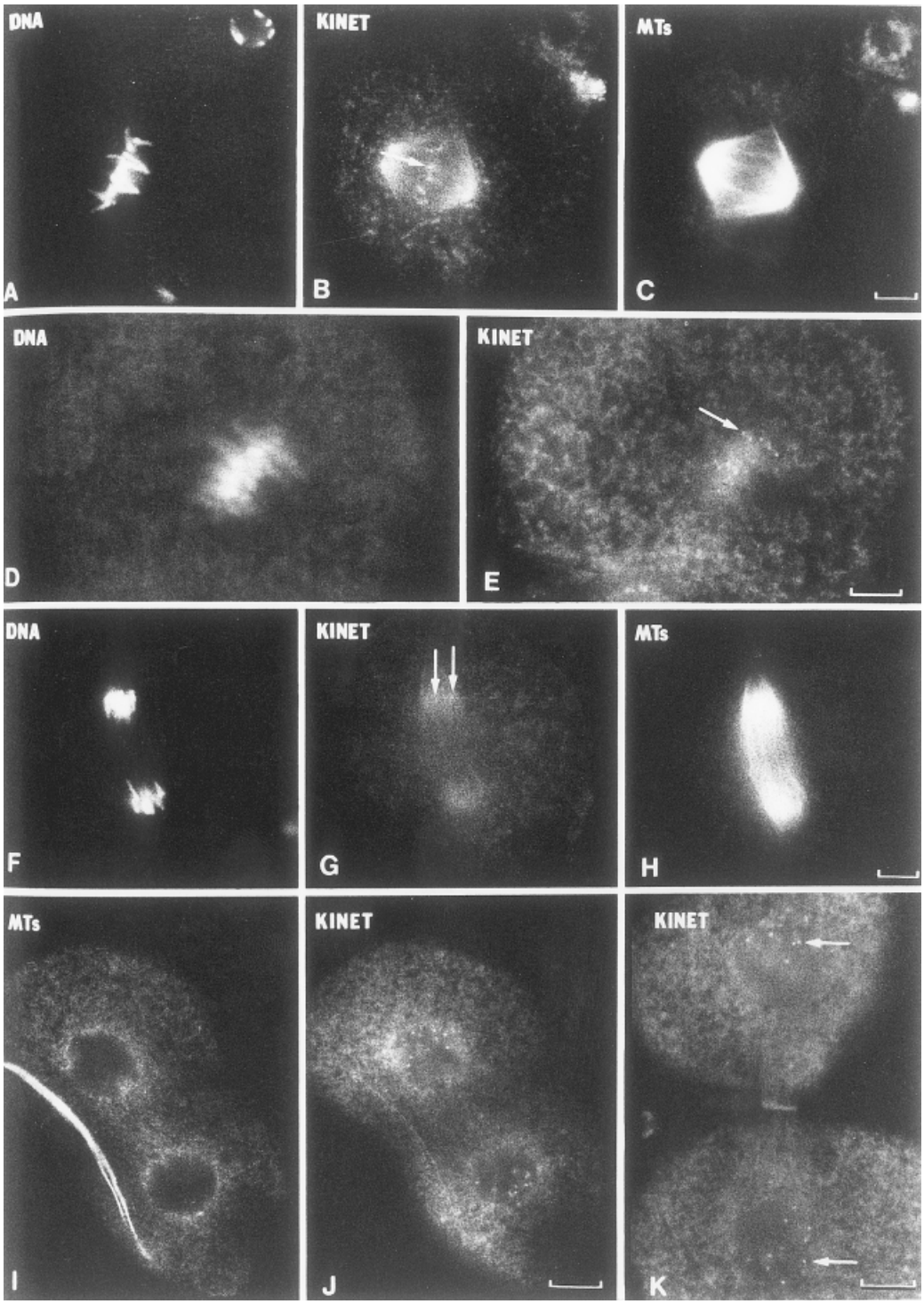

Figure Sch 4, 1988 *'Fig. 4A–K. First mitosis: metaphase through cleavage. At metaphase the chromosomes are aligned at the equator of the mitotic spindle (A, D) and the kinetochores are oriented perpendicular to the spindle axis (B, E; arrow). The microtubules of the mitotic spindle in mouse oocytes typically are anastral with wide poles (C). At anaphase (F), the intensity of kinetochore staining is greatly reduced (G). The spindle has elongated with interzonal microtubules becoming apparent (H). At cleavage (I), the kinetochores are largely restricted to the polar hemispheres of the blastomere nuclei. A–C and F–H: triple labelled for DNA (DNA), kinetochores (KINET) and microtubules (MTs); D–E: double labelled for DNA and kinetochores; I–J: double labelled for microtubules and kinetochores; K: single staining for kinetochores. Scale bars represent 10 μm.* Reprinted with permission from Schatten *et al*. (1988).

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fish eggs. Thus while there are no reports in the literature of either of these processes having been investigated in mammalian embryos, when considered in the context of conservation of process in embryology, it may not be unreasonable to entertain the possibility that they may occur.

Returning to reports from Schatten's group, what evidence may one identify in these reports to support the interpretation that a new small cell is being formed between syngamy and first cleavage? Schatten *et al.*'s, report (1988) provides images illustrating the distribution of DNA, kinetochores and microtubules in ovum and zygote. It includes images over a crucial time span, namely between early prometaphase (Fig. Sch 3J, 1988) and metaphase (Fig. Sch 4A, 1988). This is a similar time span over which Howlett & Bolton (1985) reported an apparent increase in DNA content, which they attributed to PB2. The most interesting feature of these images is not that they show stages of accumulation of DNA in an apparently emerging small cell, but the anomalous distribution of DNA in the cell and its pattern of accumulation. What we see is an increasing peripheral distribution of small clumps of DNA which take on a circular distribution in a small region of the zygote (Fig. Sch 3J, 3M and 4A, 1988). This is a very atypical means of DNA accumulation in a mammalian cell. As a comparison, if we consider the formation of PB2 (Sato & Blandau, 1979), it is clear from the outset that its DNA is located in a discrete mass. It seems, therefore, that we may be dealing with a most unusual type of cell formation for a mammal. Initially trying to resist this possible interpretation I thought the peripheral distribution of clumps of DNA, which bears a superficial resemblance to what one might expect of a cell undergoing apoptosis, might in fact be evidence of the final demise of PB2. However, the evidence which would require a rejection of this interpretation is that in any event this would still necessitate continuity in PB2 DNA, regardless of its location, over the first cell cycle. Also one would not expect the peripherally distributed quantity of DNA to be increasing, and finally one would not then expect this peripherally distributed DNA to form a large central aggregation of DNA, i.e. the nucleus evident at cleavage (Figs. 10, 11).

A more plausible explanation is at hand if we entertain the possibility that a new small cell is being formed by an atypical means involving reabsorbed PB2 DNA acting as a template for DNA replication and/or maternally inherited mRNA being the source of the DNA by a reverse transcription mechanism. A scenario involving the reabsorption of PB2 DNA might also explain the persistence of the mid-body connection between PB2 and the vitellus. If DNA is being formed in the peripheral vitelline cytoplasm then it is very likely to become attached to the cytoskeleton associated with the cell membrane. If this attachment is to a limited region of the cytoplasm, this peripherally located DNA may readily become isolated into a separate cell in the event of that portion of the cell membrane becoming pinched off. Observation of such an event, however, under the conventional light microscope, in the absence of a specific DNA stain, would

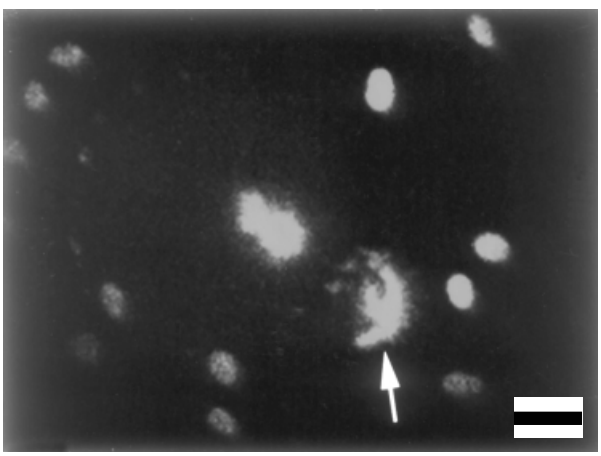

Figure 10 Confocal microscope projected images of an air-dried zygote soon after syngamy, stained with propidium iodide. While the zona pellucida does not stain, the distribution of surrounding chicken erythrocytes, used as controls, helps identify the periphery of the zygote. The erythrocytes are not in focus due to the difference in size compared with the zygote. Newly forming blastomere nuclei are located centrally, and near the periphery is a substantially stained mass indicated by the arrow. Propidium iodide is known to stain both DNA and RNA. However, given the unstable nature of RNA, particularly in air dried specimens, the staining reaction is most likely to be with DNA. The considerable size of this peripheral mass would indicate that it is lying within the vitellus and not between it and the zona pellucida. Scale bar represents 15 μm.

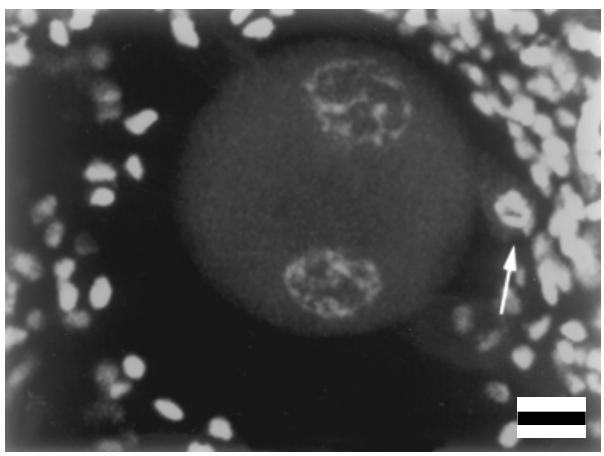

Figure 11 A confocal projected image of an air-dried zygote prior to cytokinesis. The incipient blastomere nuclei are clearly visible. So too is a distinct nucleus in a small cell, which shows a hint of a cytoplasmic connection to one of the blastomeres near the potential cleavage furrow. Scale bar represents 15 μm.

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reveal a vesicular like structure being formed, because there is as yet no central aggregation of DNA in a nucleus. This might explain why video footage of first cleavage has not alerted observers to flaws in the conventional account, the structure simply being dismissed as a vesicle which had become pinched off due to the cytoplasmic turbulence which accompanies cytokinesis at first cleavage.

To facilitate incorporation of the entire peripherally located DNA into this new small cell it would be necessary for the DNA to become localised adjacent to a very limited portion of the cell membrane. While this may in part be facilitated by activity of the cytoskeleton it may also be facilitated if the site of small cell formation were close to the site of DNA production. In the event of reabsorbed PB2 DNA or any other of the cytoplasmic constituents of PB2 forming the basis of DNA manufacture for the new small cell, it would not be surprising if the site of PB2 reabsorption and the site of small cell formation were in close proximity to one another. This is indeed what the video footage (Meiosis, 1991) appears to show and also helps to explain how some observers such as Howlett & Bolton (1985) may attribute a new small cell's DNA to PB2.

On the basis of Niu *et al*.'s (1989) report one can predict that a central nucleus is not likely to appear immediately, taking perhaps 30 min to 3 h for its formation. Reports from Schatten's group are compatible with such a delay. They show that DNA is initially detectable accumulating in a limited region of the peripheral zygotic cytoplasm at prometaphase (Fig. Sch 3J, 1988), with a distinctively circular but peripheral distribution appearing away from the main zygotic DNA at metaphase (Fig. Sch 4A, 1988). A distinctive central nucleus is clearly detectable in the small cell after cleavage (Figs. 8, 9).

It is well recognised that in eukaryotic cells depending on the stage of the cell cycle, DNA may be observed in association with kinetochores, centrioles and microtubules. Might there be evidence of these in the small cell? Once again reports from Schatten's group provide incredibly good evidence of them. It appears that there is as much evidence for the presence of kinetochores in this small cell as there is for their presence in the two incipient blastomeres (Schatten *et al*., 1988; Figs. Sch 3K, 3N and 4B). Microtubules, are also very conspicuous (Figs. Sch 3L, 3O and 4C, 1988). Surprisingly in Schatten *et al.* (1988), where kinetochores are the central issues of interest, there is no comment about their presence in this small cell at this stage of the cycle, as there was recognition of their presence during pronuclear apposition in a previous figure in the report. Perhaps this is because it is being interpreted as one and the same structure (i.e. PB2), even though the DNA, kinetochore and microtubule profile of PB2 in Sch 3A, 3B and 3C (1988), at the stage of the pronuclear membrane breaking down, is quite different to that seen in the small cell at the later stages of syngamy (Fig. Sch 4A, 4B and 4C, 1988).

Thus it may be argued that there is a basis for recognising a potential capacity for nuclear DNA formation by non-meiotic or mitotic means in the small cell seen at first cleavage in the mouse zygote.

Is there any evidence that this small cell seen at first cleavage may continue over the preimplantation period?

If this cell were to continue to the blastocyst stage located on the periphery, without undergoing division, then we might expect it to appear large compared with the size of a blastomere at the blastocyst stage due to the progressive reduction in the size of blastomeres. These proportions and the peripheral location of a cell at the blastocyst stage can clearly be seen in an illustration originating from Dr H. Pratt at Cambridge, in Horgan *et al*. (1986), though again the reader's attention is not drawn to the feature.

To avoid the cumbersome terminological problem of referring to this cell, in the event of direct experimental confirmation of its formation at first cleavage, I would suggest an alternative name such as 'the key cell' or 'K-cell', in celebration of its role in unlocking our minds from the comfortably familiar and an anticipated key role both of the cell in the preimplantation phase, and of wider understandings in cell biology.

Does the situation in mice have any relevance to humans, particularly in the use of invasive techniques in reproductive technology?

After first cleavage, morphologically the human embryo is distinctive from the mouse in both variation in the size of individual blastomeres and their nuclear content (Munne *et al*., 1995; Pelinck *et al*., 1998). Both anucleate and multinucleate (aneuploidy) states are common in cells of apparently morphologically normal human embryos (Winston *et al*., 1993). The variation in nuclear DNA appears to be so considerable that analysing a single blastomere for the purpose of PGD is increasingly being considered unreliable due to the problem of mosaicism, and polar body analysis (PB1 together with PB2) is being preferred (Munne *et al*., 1995; Strom *et al*., 1998; Rechitsky *et al*., 2001).

If we consider such variability in blastomere size and nuclear content in an evolutionary context, it is possible that a genuine multinucleate blastomere, as distinct from blastomeres in the telophase stage of mitosis, may be the result of a partial or incomplete process of the situation in the mouse. That is, the new small cell (Figs. 8, 9) nucleus once formed may either be retained within a blastomere cytoplasm or, as in the mouse, isolated into a separate small cell – a small 'blastomere'.

One of the important requirements of cloning as

practised at present is the production of a host egg devoid of nuclear DNA, into which a nucleus from the cell of an organism to be cloned will be inserted. The production of a host egg devoid of nuclear DNA inevitably results in the loss of egg cytoplasm during the removal of its nuclear DNA. Recognising this, Dominko *et al*. (2000) have tried to develop non-deleterious ways of visualising the egg DNA to minimise the loss of cytoplasm during its extraction. If the nucleus in the small cell of the early mouse embryo (Figs. 8, 9), or its equivalent in a multinucleate or mononuclear human blastomere, result from atypical methods of DNA production, which are highly dependent on cytoplasmic constituents such as mRNA (see previous discussion), then it further increases the risks of such invasive techniques.

Conclusion

The principal basis for this review has been to examine the longevity of PB2; in particular to examine whether the interpretation that the nucleated small cell in pristine condition, seen at first cleavage in the mouse and identified in the literature as PB2, is based on fact or presumption. I suggest that the weight of evidence shows such an interpretation to be based on presumption. Furthermore there are reasonable grounds for considering such a presumption to be false. There is a strong basis for seeking to determine the longevity of PB2. There is a crucial lack of evidence in the literature to demonstrate a continuity of PB2 DNA over the first cell cycle.

All in all it seems unlikely that PB2 continues past first cleavage with the integrity normally associated with a functional cell. There seems little doubt that vesicular structures or fragments of polar bodies may continue well past first cleavage. It is by no means unlikely that without the detection of a distinct nucleus or DNA staining techniques, vesicular remnants particularly of PB2 may be mistaken for an integral cell (see Figs. 8, 9). Notwithstanding the above, there is still a need to be able to describe the longevity of PB2 and the nature of its demise more definitively. Removing PB2 during the first cell cycle and observing whether a new small cell is still formed after first cleavage, will eliminate the possibility that a newly formed small cell was being confused for PB2. Using a double staining technique on fresh material, such as Hoechst and propidium iodide to stain DNA, should help determine whether all the polar bodies experience a necrotic demise or whether PB2 may experience an apoptotic one. If the latter were to be the case then it would also be our earliest evidence of apoptosis in a mammal and provide an excellent model for further investigation.

The available meagre evidence, while supportive of a case for there being more than a haploid content of DNA in the nucleated small cell seen at first cleavage, clearly highlights the need for more definitive quantification and analysis of its DNA. Some work on most of these issues is proceeding. In the event of such investigations demonstrating an anomalous mechanism of its nuclear DNA formation, the potential of this finding in understanding other cellular processes, including retrovirus replication and genetic manipulation of cellular processes, may be considerable.

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